

The genetic characterization of *Pseudomonas syringae* pv. *tagetis* based on the 16S–23S rDNA intergenic spacer regions

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Abstract

Pseudomonas syringae pv. *tagetis*, a plant pathogen being considered as a biological control agent of Canada thistle (*Cirsium arvense*), produces tagetitoxin, an inhibitor of RNA polymerase which results in chlorosis of developing shoot tissues. Although the bacterium is known to affect several plant species in the Asteraceae and has been reported in several countries, little is known of its genetic diversity. The genetic relatedness of 24 strains of *P. syringae* pv. *tagetis* with respect to each other and to other *P. syringae* and *Pseudomonas savastanoi* pathovars was examined using 16S–23S rDNA intergenic spacer (ITS) sequence analysis. The size of the 16S–23S rDNA ITS regions ranged from 508 to 548 bp in length for all 17 *P. syringae* and *P. savastanoi* pathovars examined. The size of the 16S–23S rDNA ITS regions for all the *P. syringae* pv. *helianthi* and all the *P. syringae* pv. *tagetis* strains examined were 526 bp in length. Furthermore, the 16S–23S rDNA ITS regions of both *P. syringae* pv. *tagetis* and *P. syringae* pv. *helianthi* had DNA signatures at specific nucleotides that distinguished them from the 15 other *P. syringae* and *P. savastanoi* pathovars examined. These results provide strong evidence that *P. syringae* pv. *helianthi* is a nontoxigenic form of *P. syringae* pv. *tagetis*. The results also demonstrated that there is little genetic diversity among the known strains of *P. syringae* pv. *tagetis*. The genetic differences that do exist were not correlated with differences in host plant, geographical origin, or the ability to produce toxin.

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1. Introduction

The characterization of a plant pathogen with respect to other closely related plant diseases and isolates of the same pathovar is important to the commercialization of any microbial biological control agent of weeds, particularly if the disease is known to occur in nontarget plants or if different isolates are to be developed for different

target plants. The plant pathogen *Pseudomonas syringae* pv. *tagetis* is of interest as a biological control agent of Canada thistle [*Cirsium arvense* (L.) Scop.] and other weeds in the Asteraceae family (Abbas et al., 1999; Gronwald et al., 2002, 2003; Hoeft et al., 2001; Johnson and Wise, 1991; Johnson et al., 1996; Tichich and Doll, 2003). The disease has been reported in at least six different countries and has been isolated from at least eight different species in the Asteraceae, two of which are crops, sunflower (*Helianthus annuus* L.) and Jerusalem artichoke (*Ambrosia tuberosa* L.) (see Kong et al., 2003). Consequently, a thorough characterization of this pathovar with respect to its hosts and country of origin is important in assessing the risks associated with using

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P. syringae pv. *tagetis* as a biological control agent for weeds.

Based on DNA hybridization analysis, Gardan et al. (1999) determined that *P. syringae* pv. *tagetis* and *P. syringae* pv. *helianthi* are genetically distinct from other *P. syringae* pathovars. However, only two isolates of *P. syringae* pv. *tagetis* were included in the study. Using polymerase chain reaction (PCR) analysis to amplify genes required for tagetitoxin production, Kong et al. (2003) demonstrated that isolates of *P. syringae* pv. *tagetis* could be distinguished from other closely related *P. syringae* pathovars except for *P. syringae* pv. *helianthi*. However, the latter study did not examine what genetic differences may exist among *P. syringae* pv. *tagetis* isolates originating from different countries and recovered from different host plants. To increase our knowledge of the genetic diversity within the pathovar and of its phylogenetic relationship to other *P. syringae* pathovars, we compared the 16S–23S rDNA intergenic spacer (ITS) regions of 17 closely related *Pseudomonas* pathovars and 24 strains of *P. syringae* pv. *tagetis*. The results of that study are reported here.

2. Materials and methods

2.1. Bacterial strains and DNA isolation

The *P. syringae* and *Pseudomonas savastanoi* strains used in this study (Table 1) were grown at 28°C in King's medium B (KB) (King et al., 1954) in broth or solid state. *Escherichia coli* JM 109 was used as a host strain for cloning and was grown on Luria–Bertani liquid medium (Sambrook et al., 1989) at 37°C. Total DNA from bacteria was isolated using a genomic DNA isolation kit (MBI Fermentas, Hanover, MD) and further purified using standard chloroform–phenol extraction procedures (Sambrook et al., 1989).

2.2. PCR amplification of 16S–23S rDNA ITS regions

PCR amplifications were performed in a total of 25 µl. Reaction mixtures consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.8 µM each primer, 200 µmol each dNTP, 1 U AmpliTaq Gold (Perkin–Elmer, Norfolk, CT), and 40 ng of bacterial DNA. The reactions were performed in a Perkin–Elmer GeneAmp PCR System 9600. Denaturation was at 95°C for 10 min for the first cycle and 94°C for 30 s for each subsequent cycle. Annealing and elongation were at 58°C for 20 s and 72°C for 30 s, respectively, for 5 cycles adding 2 s to the annealing time per cycle. Annealing and elongation for an additional 25 cycles were at 58 and 72°C for 30 s adding 1 s to the annealing and extension times per cycle. Reactions were terminated after a final 5 min elongation at 72°C. The amplicons in the reaction mix were sepa-

rated by electrophoresis in a 0.8% agarose gel. The PCR primers (space F-B and space R) used in the PCR amplifications of the 16S–23S rDNA ITS regions were those described by Sawada et al. (1997). The primers correspond to conserved sequences at the 3' end of the 16S rDNA from position 1486 to position 1511 and the 5' end of 23S rDNA from position 50 to position 26 based on *E. coli* numbering (Gutell et al., 1994).

2.3. Cloning and sequencing of PCR amplicons

PCR amplicons were excised from electrophoresis gels and purified using the Qiaex II Gel extraction kit (Qiagen, Valencia, CA). The purified DNA fragments were ligated into the pGEM-T Easy vector and cloned into *E. coli* JM109 following the manufacturer's recommendations for the pGEM-T Easy Vector Systems II (Promega, San Luis Obispo, CA). The cloned PCR products were sequenced by Big Dye terminator reactions with primers T7 and SP6 to cover both strands, and performed with an ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA).

2.4. DNA sequence analysis

The multiple sequence alignment of the DNA sequences was constructed using the Clustal method within DNASTAR software (DNASTAR, Madison, WI). The phylogenetic tree was generated by the Neighbor-Joining algorithm using the PHYLIP program, version 3.6 (Felsenstein, 2002). Branch robustness was evaluated using 1000 bootstrap replicates. The bootstrap consensus phylogenetic tree generated was visualized using the TREEVIEW program, version 1.6.6 (Page, 1996). The 16S–23S rDNA ITS sequences were deposited with GenBank Accession Nos. AY342162–AY342210 and AY835932–AY835935.

2.5. Bioassays for apical chlorosis

Sunflower plants (*H. annuus* cv. 'Autumn Beauty') were grown in 50-cell flats containing Jiffy Mix Plus (Jiffy Products of America, Batavia, IL) at 24°C under 600 µmol m⁻² s⁻¹ for a 16 h photoperiod. After 7 days, plants were inoculated by stabbing them just below the cotyledons with a 25 gauge hypodermic needle carrying cells from a 2-day-old bacterial culture grown at 28°C on King's medium B agar. Sunflower plants were evaluated for apical chlorosis 5–7 days after inoculation. Bioassays were repeated at least three times for strains that proved nontoxic.

3. Results and discussion

The size of the 16S–23S rDNA ITS sequences ranged from 508 bp (*P. syringae* pv. *atrofaciens*) to 548 bp

Table 1

Pseudomonas pathovars and strains used in this study

Species	Host/common name	Country of origin	Source ^a
<i>Pseudomonas savastanoi</i>			
pv. <i>glycinea</i> 4180	<i>Glycine max</i> L. Merr./soybean	New Zealand	Bender
pv. <i>phaseolicola</i> 19304† ^b	<i>Phaseolus vulgaris</i> L./bean	Canada	ATCC
<i>Pseudomonas syringae</i>			
pv. <i>actinidiae</i> 9617†	<i>Actinidia deliciosa</i> (Chev.)/kiwi	Japan	ICMP
pv. <i>antirrhini</i> 4303†	<i>Antirrhinum majus</i> L./snap dragon	UK	ICMP
pv. <i>aptata</i> 4387	<i>Beta vulgaris</i> L./sugar beet	USA	ICMP
pv. <i>atrofaciens</i> 4394†	<i>Triticum aestivum</i> L./wheat	Hungary	ICMP
pv. <i>coronafaciens</i> 3113†	<i>Avena sativa</i> L./oat	UK	ICMP
pv. <i>delphinii</i> 529†	<i>Delphinium</i> sp./delphinium	New Zealand	ICMP
pv. <i>garcae</i> 4323†	<i>Coffea arabica</i> /coffee	Brazil	ICMP
pv. <i>helianthi</i> 3549	<i>Helianthus annuus</i> L./sunflower	New Zealand	ICMP
pv. <i>helianthi</i> 3922	<i>Helianthus annuus</i> L./sunflower	Zambia	ICMP
pv. <i>helianthi</i> 4531†	<i>Helianthus annuus</i> L./sunflower	Mexico	ICMP
pv. <i>helianthi</i> 10564	<i>Helianthus annuus</i> L./sunflower	Yugoslavia	ICMP
pv. <i>helianthi</i> 11933	<i>Helianthus annuus</i> L./sunflower	France	ICMP
pv. <i>maculicola</i> #10	<i>Brassica oleracea</i> L./cauliflower	USA	Hutcheson
pv. <i>pisi</i> 2452†	<i>Pisum sativum</i> L./pea	New Zealand	ICMP
pv. <i>papulans</i> 4048†	<i>Malus domestica</i> (Borkh.) Borkh./apple	USA	ICMP
pv. <i>syringae</i> B1631†	<i>Syringa vulgaris</i> L./common lilac	UK	ARSCC
pv. <i>tabaci</i> 2835†	<i>Nicotiana tabacum</i> L./tobacco	Hungary	ICMP
pv. <i>tagetis</i> 1-0392	<i>Tagetes erecta</i> L./marigold	USA	Johnson
pv. <i>tagetis</i> 1-502a M2	<i>Cirsium arvense</i> (L.) Scop./Canada thistle	USA	Johnson
pv. <i>tagetis</i> 1-1065x	<i>Ambrosia trifida</i> L./giant ragweed	USA	Johnson
pv. <i>tagetis</i> 1-1332a	<i>Helianthus annuus</i> /sunflower	USA	Johnson
pv. <i>tagetis</i> 1-1394	<i>Ambrosia artemisiifolia</i> L./common ragweed	USA	Johnson
pv. <i>tagetis</i> 1-2386	<i>Ambrosia artemisiifolia</i> /common ragweed	USA	Johnson
pv. <i>tagetis</i> 4091†	<i>Tagetes erecta</i> /marigold	Zimbabwe	ICMP
pv. <i>tagetis</i> 5866	<i>Tagetes erecta</i> /marigold	Australia	ICMP
pv. <i>tagetis</i> 6369	<i>Tagetes erecta</i> /marigold	Australia	ICMP
pv. <i>tagetis</i> 6371	<i>Tagetes</i> sp./marigold	USA	ICMP
pv. <i>tagetis</i> 6564	<i>Tagetes erecta</i> /marigold	USA	ICMP
pv. <i>tagetis</i> 26808	<i>Tagetes erecta</i> /marigold	Australia	ACPPB
pv. <i>tagetis</i> 26816	<i>Tagetes erecta</i> /marigold	Australia	ACPPB
pv. <i>tagetis</i> 43127	<i>Taraxacum officinale</i> G.H. Weber ex Wiggers/common dandelion	USA	ATCC
pv. <i>tagetis</i> 43128	<i>Ambrosia artemisiifolia</i> /common ragweed	USA	ATCC
pv. <i>tagetis</i> 349392	<i>Cynara scolymus</i> L./globe artichoke	New Zealand	CABI
pv. <i>tagetis</i> 349393	<i>Helianthus tuberosus</i> L./Jerusalem artichoke	New Zealand	CABI
pv. <i>tagetis</i> APC5	<i>Helianthus annuus</i> /sunflower	South Africa	Fourie
pv. <i>tagetis</i> APC17	<i>Helianthus annuus</i> /sunflower	South Africa	Fourie
pv. <i>tagetis</i> APC19	<i>Helianthus annuus</i> /sunflower	South Africa	Fourie
pv. <i>tagetis</i> APC20	<i>Helianthus annuus</i> /sunflower	South Africa	Fourie
pv. <i>tagetis</i> APC22	<i>Helianthus annuus</i> /sunflower	South Africa	Fourie
pv. <i>tagetis</i> EB037	<i>Ambrosia artemisiifolia</i> /common ragweed	USA	Johnson
pv. <i>tagetis</i> M1-1	<i>Helianthus annuus</i> /sunflower	USA	Fett
pv. <i>tomato</i> 2844†	<i>Lycopersicon esculentum</i> Mill./tomato	UK	ICMP

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^b †, Pathotype.

(*P. syringae* pv. *pisi*) for the 23 *P. syringae* pathotypes examined. As reported by Sawada et al. (1997), the 16S–23S rDNA ITS region of the *P. syringae* and *P. savastanoi* pathovars includes the transfer RNA sequences for tRNA^{Ile} and tRNA^{Ala}, which are highly conserved, and major areas of sequence variation immediately before and after the tRNA^{Ala} region (Fig. 1). The results of the phylogenetic relationships of the *P. syringae* pathovars and closely related species based on 16S–23S rDNA ITS sequence analysis were similar to that based on DNA hybridization analysis (Gardan et al., 1999) (Fig. 2). Our results differed from Gardan et al. (1999) in that the 16S–23S rDNA ITS sequence results indicated that *P. syringae* pv. *papulans* and *P. syringae* pv. *pisi* are more closely related to the genomospecies group 7 than the other *P. syringae* pathovars of genomospecies 1 that were included in this study.

Only the pathovars, *P. syringae* pv. *tagetis* and *P. syringae* pv. *helianthi*, had 16S–23S rDNA ITS sequences of 526 bp (Fig. 1). The 16S–23S rDNA ITS regions of the *P. syringae* pv. *tagetis* type strain differed by only 3 bp from the *P. syringae* pv. *helianthi* type strain, as compared to a difference of 16–18 bp for the other closely related pathovars (*P. syringae* pv. *aptata*, *P. syringae* pv. *garcae*, *P. syringae* pv. *tabaci*, *P. syringae* pv. *syringae*, *P. syringae* pv. *coronafaciens*, and *P. savastanoi* pv. *glycinea*) (Fig. 1). Consequently, and similar to DNA hybridization results reported by Gardan et al. (1999), *P. syringae* pv. *tagetis* and *P. syringae* pv. *helianthi* clustered together yet separate from the other *P. syringae* pathovars (Fig. 2). Symptoms, excluding apical chlorosis, as well as biochemical and nutritional indicators are nearly identical for *P. syringae* pv. *tagetis* and *P. syringae* pv.

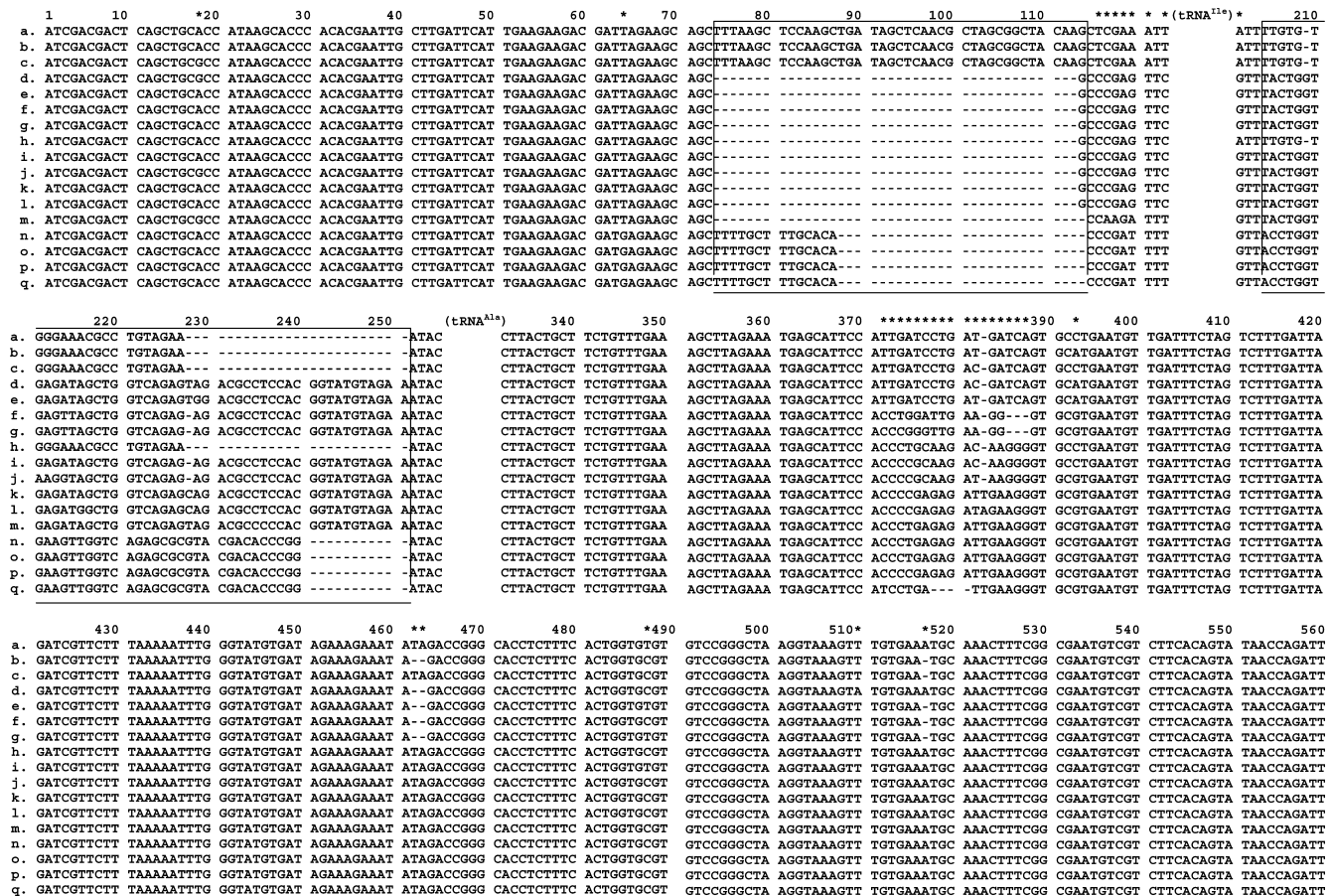


Fig. 1. Total sequence alignment of the 16S–23S rDNA intergenic spacer regions of *Pseudomonas syringae* and *Pseudomonas savastanoi* pathovars. Rows: a, *P. syringae* pv. *pisi* 2452; b, *P. syringae* pv. *phaseolicola* 19304; c, *P. syringae* pv. *papulans* 4048; d, *P. syringae* pv. *glycinea* 4180; e, *P. syringae* pv. *tabaci* 2835; f, *P. syringae* pv. *helianthi* 4531; g, *P. syringae* pv. *tagetis* 4091; h, *P. syringae* pv. *atrofaciens* 4394; i, *P. syringae* pv. *aptata* 4387; j, *P. syringae* pv. *syringae* B1631; k, *P. syringae* pv. *garcae* 4323; l, *P. syringae* pv. *coronafaciens* 3113; m, *P. syringae* pv. *delphinii* 529; n, *P. syringae* pv. *maculicola* 10; o, *P. syringae* pv. *tomato* 2844; p, *P. syringae* pv. *antirrhini* 4303; and q, *P. syringae* pv. *actinidae* 9617. Asterisks indicate nucleotide differences and deletions indicated by dashes. The highly variable regions between positions 74–114 and 204–251 are enclosed in boxes. Nucleotides 561–574 are not shown but were identical (GCTTGGGGTTATAT) for all the strains tested. The sequences of the highly conserved transfer RNA genes tRNA^{Ile} and tRNA^{Ala} are not presented, however, their locations within the 16S–23S rDNA ITS are indicated.

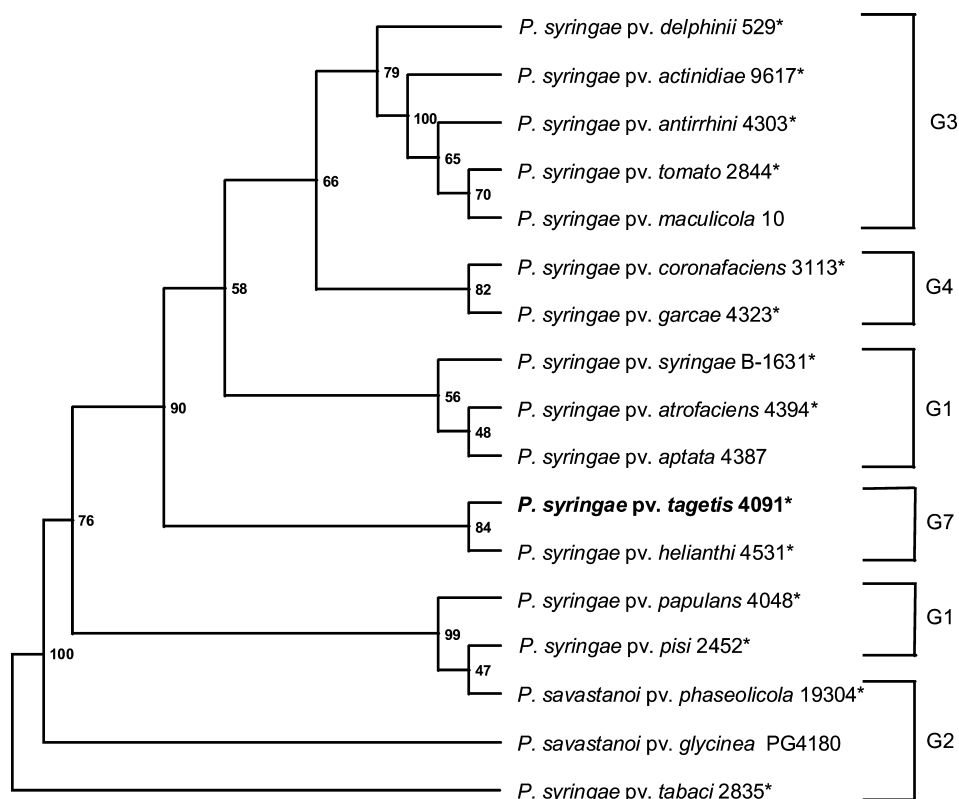


Fig. 2. Phylogenetic tree based on the 16S–23S rDNA intergenic spacer regions of 17 *Pseudomonas syringae* and *Pseudomonas savastanoi* pathovars. The bootstrap consensus phylogeny tree was generated using the Neighbor-Joining algorithm and the percentages of bootstrap replicates (based on 1000 bootstrap samples) are placed at the tree nodes. The asterisk following pathovar names indicates pathotype. G values to the right of the brackets indicate genomospecies groups in which the pathovars were previously assigned by Gardan et al. (1999).

helianthi (Arsenijević et al., 1994; Fourie and Viljoen, 1994; Gulya and Bantari, 1982; Kùdela and Zacha, 1998; Laberge and Sackston, 1986; Shane and Baumer, 1984; Trimboli et al., 1978). Based on results of the PCR analysis of *P. syringae* pathovars using PCR primer sets developed from genes required for tagetitoxin production, Kong et al. (2003) suggested that *P. syringae* pv. *helianthi* could possibly be a nontoxigenic form of *P. syringae* pv. *tagetis*. In fact, the 16S–23S rDNA ITS sequences for all five isolates of *P. syringae* pv. *helianthi* examined were identical to one or more of the 16S–23S rDNA ITS sequences of a worldwide collection of *P. syringae* pv. *tagetis* (Table 2). This is new evidence that supports the conclusion of Kong et al. (2003) that *P. syringae* pv. *helianthi* is a nontoxigenic form of *P. syringae* pv. *tagetis*.

Most importantly, the 16S–23S rDNA ITS analysis of a worldwide collection of 24 *P. syringae* pv. *tagetis* strains that included isolates from all known host plants demonstrated that there is very little genetic diversity within this pathovar. For example, 23 of the 24 strains examined differed from each other by no more than 3 nucleotides in this ITS region (Table 2). The exception, *P. syringae* pv. *tagetis* 1-2386, only

differed from this by one more nucleotide difference. The 24 strains fall almost evenly into two main clusters, those with the nucleotides of cytosine (C) and guanine (G) at positions 374 and 377, and those with the nucleotides thymine (T) and adenine (A) at positions 374 and 377. Assuming that *P. syringae* pv. *helianthi* is a nontoxigenic form of *P. syringae* pv. *tagetis*, these nucleotide/position pairs are distinct for *P. syringae* pv. *tagetis*, as none of the other *P. syringae* or *P. savastanoi* pathovars had these combinations of nucleotides at the respective base-pair positions. Furthermore, what little genetic difference that does exist among these *P. syringae* pv. *tagetis* strains is not correlated with differences in host plant, geographical origin, or toxin production (Table 2).

While previous studies have shown that particular DNA regions are of a size specific to isolates of *P. syringae* pv. *tagetis* (Kong et al., 2003), this is the first study to clearly demonstrate the genetic relatedness of isolates within *P. syringae* pv. *tagetis*. Whether the lack of genetic diversity within this *P. syringae* pathovar is reflected in a similar lack of host specificity or biological activity of the strains examined has not been determined.

Table 2

Nucleotide signatures identified for *Pseudomonas syringae* pv. *tagetis* and *P. syringae* pv. *helianthi*

Species	Nucleotide position				Host plant	Origin	Apical chlorosis
	18	374	377	488			
<i>P. syringae</i> pv. <i>tagetis</i> 1-0392	A	C	G	C	Marigold	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 1065x	A	C	G	C	Giant ragweed	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 1-1394	A	C	G	C	Common ragweed	USA	–
<i>P. syringae</i> pv. <i>tagetis</i> 1-502a M2	A	C	G	T	Canada thistle	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 1-1332a	A	C	G	T	Sunflower	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 4091 ^a	A	C	G	T	Marigold	Zimbabwe	+
<i>P. syringae</i> pv. <i>tagetis</i> 6369	A	C	G	T	Marigold	Australia	+
<i>P. syringae</i> pv. <i>tagetis</i> 6564	A	C	G	T	Marigold	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 26816	A	C	G	T	Marigold	Australia	+
<i>P. syringae</i> pv. <i>tagetis</i> APC5	A	C	G	T	Sunflower	South Africa	+
<i>P. syringae</i> pv. <i>tagetis</i> APC20	A	C	G	T	Sunflower	South Africa	+
<i>P. syringae</i> pv. <i>tagetis</i> APC22	A	C	G	T	Sunflower	South Africa	+
<i>P. syringae</i> pv. <i>tagetis</i> 1-2386	G	T	A	C	Common ragweed	USA	–
<i>P. syringae</i> pv. <i>tagetis</i> 6371	A	T	A	C	Marigold	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 26808	A	T	A	C	Marigold	Australia	+
<i>P. syringae</i> pv. <i>tagetis</i> 43127	A	T	A	C	Common dandelion	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 43128	A	T	A	C	Common ragweed	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> 349392	A	T	A	C	Globe thistle	New Zealand	+
<i>P. syringae</i> pv. <i>tagetis</i> 349393	A	T	A	C	Jerusalem artichoke	New Zealand	+
<i>P. syringae</i> pv. <i>tagetis</i> EB037	A	T	A	C	Common ragweed	USA	+
<i>P. syringae</i> pv. <i>tagetis</i> APC17	A	T	A	C	Sunflower	South Africa	–
<i>P. syringae</i> pv. <i>tagetis</i> APC19	A	T	A	C	Sunflower	South Africa	–
<i>P. syringae</i> pv. <i>tagetis</i> 5866	A	T	A	C	Marigold	Australia	+
<i>P. syringae</i> pv. <i>helianthi</i> 3922	A	T	A	C	Sunflower	Zambia	–
<i>P. syringae</i> pv. <i>helianthi</i> 4531 ^a	A	T	A	C	Sunflower	Mexico	–
<i>P. syringae</i> pv. <i>helianthi</i> 10564	A	T	A	C	Sunflower	Yugoslavia	–
<i>P. syringae</i> pv. <i>helianthi</i> 3549	A	T	A	T	Sunflower	New Zealand	–
<i>P. syringae</i> pv. <i>helianthi</i> 11933	A	T	A	T	Sunflower	France	–
<i>P. syringae</i> pv. <i>tagetis</i> M1-1	A	T	A	T	Jerusalem artichoke	USA	+

^a Pathotype for the pathovar.

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